

APPENDIX C

PRODUCTION OF HUMAN TISSUE PLASMINOGEN ACTIVATOR IN TRANSGENIC MOUSE MILK

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We set out to express an exogenous gene in the mammary epithelium of transgenic mice in the hope that the encoded protein would be secreted into milk. The promoter and upstream regulatory sequences from the murine whey acid protein (WAP) gene were fused to cDNA encoding human tissue plasminogen activator (t-PA) with its endogenous secretion signal sequence. This hybrid gene was injected into mouse embryos, resultant transgenic mice were mated, and milk obtained from lactating females was shown to contain biologically active t-PA. This result establishes the feasibility of secretion into the milk of transgenic animals for production of biologically active heterologous proteins, and may provide a powerful method to produce such proteins on a large scale.

Genes injected into mouse embryos may be incorporated into the germ line and be expressed in patterns that mimic those of their endogenous counterparts^{1,2}. The pattern of spatial and temporal expression of foreign genes in transgenic animals can be controlled by prior manipulation of the signals regulating gene expression. We introduced into mice a construct designed to express a foreign protein in the lactating mammary epithelium in which 5' sequences from the whey acid protein gene were fused with a cDNA coding for tissue plasminogen activator. We demonstrate here that such an approach is a feasible means of expression of foreign proteins into secreted milk.

Whey acid protein (WAP) is the most abundant whey protein in mouse milk³. During lactation, the level of WAP RNA in the mammary gland increases approximately 340-fold from the barely detectable levels present in the mammary gland of virgin mice⁴, and accumulates in lactating tissue at levels of about 15% of the total mRNA⁴⁻⁶. Expression of the WAP gene and the stabilization of its mRNA are subject to complex regulation by both steroid and peptide hormones⁴, and putative regulatory protein binding sites within the WAP promoter have been described⁷. Since WAP is found in mouse milk at high levels and the gene had been previously cloned and

characterized^{8,9}, we chose to utilize WAP upstream DNA as a promoter in our expression vector. By demonstrating secretion of a foreign protein into milk, the results reported here extend earlier observations showing upstream sequences from the WAP gene were able to target gene expression to the lactating mammary gland in transgenic mice¹⁰.

RESULTS AND DISCUSSION

Construction of t-PA expression vector. A mammary expression vector was constructed in which 5' sequences from the whey acid protein gene were fused with a cDNA coding for tissue plasminogen activator. t-PA has great potential clinical utility as an agent to dissolve fibrin clots and thus treat victims of myocardial infarction and other life threatening conditions. Its advantage relative to other pharmacological agents such as streptokinase and urokinase lies in its specificity for fibrin. Moreover, assay of its biological activity is both sensitive and convenient and an antibody kit is available for routine screening.

The t-PA gene utilized here was a cDNA clone from a human uterus cDNA library. The t-PA DNA sequence was determined previously and the protein expressed in C127 cells using bovine papilloma vectors¹¹. The construct shown in Figure 1 (designated WAP-tPA) is a tripartite fusion consisting of 2.6 kb of upstream DNA from the WAP gene through the endogenous CAP site, t-PA cDNA beginning in the untranslated 5' region, and the polyadenylation/termination signals from SV40. This tPA/SV40 polyadenylation cassette was characterized previously¹¹. The secretion signal sequence in this construct derives from the native t-PA gene; the analogous signal encoding region from the WAP gene was removed in the construction. We did not know *a priori* whether the t-PA secretion signal would function efficiently in native mammary epithelial cells. However, since many proteins with different signal peptides are secreted efficiently by mammary cells and since milk proteins can be efficiently transported by membrane systems from other cells¹², it seemed likely that no specific signal sequence is required for secretion in mammary tissue.

Transient expression in tissue culture. To test WAP-tPA for its ability to specify production of secretable t-PA in mammary epithelial cells, the fusion gene was transfected into the mammary cell line, MCF7. Tissue culture supernatants collected 48 hours after transfection were loaded into wells of an assay plate as shown in Figure 2A. The assay consisted of lysis (clearing) of an artificial fibrin clot laid down as a matrix in agarose poured into the wells of a tissue culture plate. The degree of clearing, determined by estimating the diameter of the cleared ring

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emanating from the sample loading well, indicates the amount of active t-PA in the sample. In five repetitions from two separate transfections, the level of t-PA secreted into the culture medium was found to be 2.5, 1.5, 10, 5, and 5 ng/ml. The same five samples were also assayed by ELISA using a polyclonal anti-human t-PA antibody. By this assay, the expression levels were either below the detection limit (approximately 2 ng/ml) or 11, 10, and 10 ng/ml, respectively. Thus, whether assayed by biological activity or immunologically, MCF7 cells transfected with WAP-tPA were able to secrete t-PA.

Generation of transgenic animals. The plasmid WAP-tPA was injected into one-cell pronuclear mouse embryos as a purified Hind-3/BamHI fragment containing no procaryotic sequences. The injected embryos were implanted into pseudopregnant females and 29 mice were born. Of these, seven were identified as being transgenic by diagnostic Southern blot hybridization with a human cDNA t-PA probe. Under conditions of high stringency, this probe does not hybridize with the endogenous mouse t-PA gene. The blot patterns of three positive mice, #wt1-26, wt1-25 and wt1-7 are shown in Figure 1. By comparison to the hybridization intensity obtained with positive controls, the number of copies of the injected fragment present in the genomes of these transgenic mice was estimated to be between 20 and 50. Digestion with SacI (lanes b-d) yielded a diagnostic band of 1.75 kb that spans the WAP and t-PA junction and hybridizes to the probe (Fig. 1). The intact plasmid digested with SacI was used as a positive control for this digest (lane a). Exogenous DNA injected into embryos tends to form concatamers even when introduced as a fragment with non-cohesive ends. The 2.3 kb band seen in lanes b-d corresponds to the 3' end of the t-PA gene (which does not contain SacI sites), apparently ligated to the 5' end of the WAP promoter, and through to the first SacI site in the WAP DNA. The presence and size of this fragment is diagnostic of head-to-tail concatamers.

The EcoRI digest (control, lane h; experimentals, lane i-k) showed the expected 472 bp band internal to the t-PA gene. In addition, a 3.3 kb band can be seen that represents the 5' region of the t-PA gene and extends through the WAP gene to 5' boundary EcoRI site. Thus, despite the fact that the WAP EcoRI site was near the end of the injected fragment, it appeared to be intact in the genomic DNA of this transgenic animal. The 1.2 kb band represents the 3'-most region of the t-PA gene, which must have ligated head-to-tail to the 5' end of the WAP gene, leaving the t-PA gene bounded on its 3' end by an EcoRI site. Interestingly, the weak 2.3 kb band indicates that some of the copies of the fragments formed concatamers in a head-to-head configuration. KpnI digestion (lanes e-g) produced a single band of 4.9 kb, as expected. It is impossible to determine from this Southern blot whether all copies of the concatomer integrated at a single or at multiple sites.

Expression of biologically active t-PA in milk. Mice #wt1-26 and wt1-25 were mated to wild type males and had no apparent difficulty in conception or maintenance of pregnancy. Several days after parturition, milk was obtained from the females and was assayed for t-PA activity. Since wt1-11 was a male, it was necessary to obtain transgenic female progeny, mate them, and obtain milk from the second generation females after parturition. We have characterized expression from one progeny animal of this lineage, wt2-102. Since it was not known whether the mouse milk itself would interfere in the fibrin clot assay, we used standards consisting of recombinant t-PA added to milk from nontransgenic mice. As shown in Figure 2b, dilution of standards in milk did not affect the

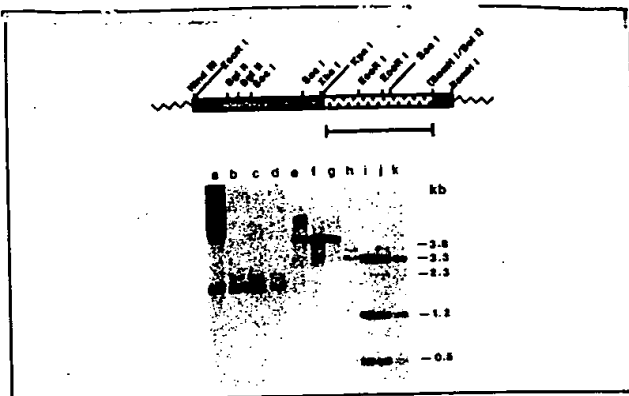


FIGURE 1 Generation of transgenic animals. Top portion: Restriction map of WAP-tPA; Bottom portion: Southern blot of DNA from tails. Lanes a and h show 500 pg of WAP-tPA DNA digested with SacI or EcoRI, respectively. Lanes b, c, and d contain 5 µg of DNA from mouse wt1-26, wt1-25 and wt1-7, respectively, digested with SacI. Lanes e-g are from mouse tails of wt1-26, wt1-25 and wt1-7, respectively, cut with KpnI, and lanes i-k are these DNAs cut with EcoRI. Lanes a-d were run on a separate gel than the rest of the lanes. Negative control DNAs did not show any hybridization to this probe under these conditions (not shown).

apparent concentration (in comparison to standards diluted in PBS), nor was there background clearing in the negative control sample wells. In this figure it can be seen that milk from wt1-26 cleared the fibrin clot to a significant extent. By comparison with lysis catalyzed by known amounts of added t-PA, the concentration was calculated to be about 200 ng/ml. In parallel assays, milk obtained from wt1-25 and wt2-102 was shown to contain 200 ng/ml and 400 ng/ml of t-PA (data not shown). When plates were incubated longer than 24 hours, minor clearing was seen in control wells containing milk from untransfected mice, but this was always significantly less than clearing seen from milk of any of the transgenic lineages. The origin of the residual fibrinolytic activity in non-transgenic mouse milk is not known. However, the presence of low levels of plasminogen activator (PA) in the lactating mammary gland of rodents¹⁵ raises the possibility that some fibrinolytic protein is present naturally in milk.

Milk from mice wt1-26, wt1-25 and 2-102 was assayed by ELISA using an anti-human t-PA polyclonal antibody (Fig. 3). A standard curve was generated by addition of known amounts of human t-PA to mouse milk. Identical curves were generated by dilution of t-PA in cow milk and aqueous buffer (not shown). The inset of Figure 3 shows results of an assay of serial dilutions of milk from wt1-26 confirming that about 300 ng/ml of t-PA was present in this sample. Milk from wild type mice showed no signal in the ELISA. Milk from wt1-25 and wt2-102 contained t-PA at concentrations of 114 and 460 ng/ml, respectively (data not shown). The measurements by fibrin clot lysis and ELISA were not sufficiently accurate to determine precisely the specific activity of the t-PA produced in milk. Further studies of the purified protein (now in progress) will establish whether the specific activity of the protein is identical to that produced by melanoma cells and by recombinant DNA methods. Pilot studies indicate that t-PA remains stable and bioactive in whole milk for at least 48 hours at 37°C, and can be stored at -80°C (data not shown).

Since WAP RNA constitutes as much as 15% of the poly A (+) mRNA in the lactating mammary gland, it is probable that the level of t-PA in the milk of these mice is far below the level of endogenous whey acid protein. This

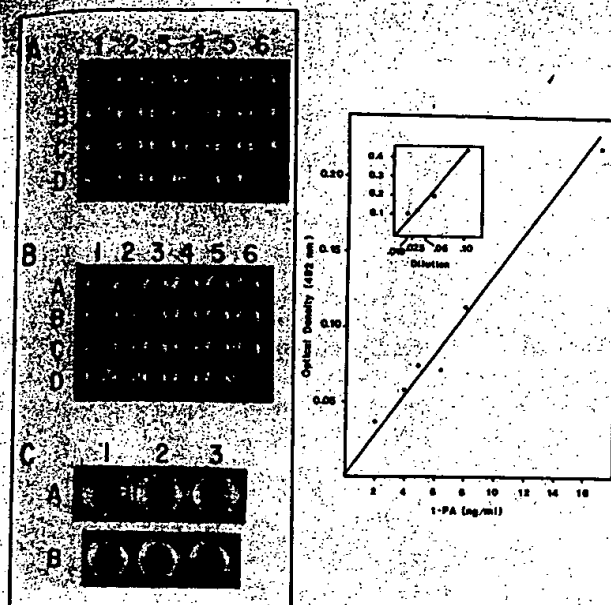


FIGURE 2 Clot lysis bioassay of secreted t-PA. (A) Transient expression of WAP t-PA in MCF7 cells. Two transfections were done, #1 (two repetitions, a and b) and #2 (3 repetitions, a, b, and c). In row A, columns 1 through 6 are recombinant DNA t-PA standards diluted in culture medium at concentrations of 20, 10, 5, 2.5, 1.25, and 0 ng/ml. Row B contains samples from transfection #1. In columns 1, 2, and 3 are three dilutions of sample from one transfection: 1×, .5×, and .25×, respectively. In columns 4, 5, and 6 of row B are similar dilutions from the repeat transfection. Row C, columns 1–3, row C, columns 4–6; and row D, columns 1–3 contain similar dilutions from the three repetitions of transfection #2. (B) Milk from transgenic mouse #wt1-26. Following identification of mouse #wt1-26 as a positive transgenic, the mouse was mated to a wild type male. Seventeen days after the first litter was born, milk was removed from the lactating female following stimulation with oxytocin. Milk was diluted in PBS by 50% and stored frozen. Milk was diluted further in PBS as indicated below just prior to assay and added to the wells of a fibrin clot lysis plate. The positive controls were generated by addition of recombinant t-PA to media composed of either 10% negative mouse milk (row A), 10% negative cow milk (row B), or PBS (row C). Concentrations of t-PA in the milk dilution curves, from columns 1 through 5 are: 40, 20, 10, 5, and 0 ng/ml. The concentrations in the PBS dilution curve, from column 1 through 6 are: 40, 20, 10, 5, 2.5, and 0 ng/ml. In row A, column 6 is the milk from mouse #wt1-26 at a final concentration of 10% and in row B, column 6 is the milk at a concentration of 5%. This photograph was taken after approximately 8 hours of assay incubation time. The negative mouse milk used for these controls was pooled from outbred CD-1 mice in different stages of lactation. In other negative controls (not shown) milk was used from inbred females of the same strain used for microinjection and was obtained at the same stage of lactation as the positive sample. The specificity of recombinant t-PA secreted into mouse milk was shown to be plasminogen in other experiments (not shown) in which plasminogen was omitted from the agarose matrix in similar fibrin clot lysis assays. (C) Enlargement of key data of sections A and B. Row A shows an enlargement of the data of section A (above), row C, columns 1–3; Row B shows one of the data of section B (above), row A, columns 4–6.

FIGURE 3 Quantitation by ELISA of recombinant t-PA secreted into milk of mouse wt1-26. The standard curve was performed in negative mouse milk diluted to a final concentration of 10% with PBS, to which was added t-PA supplied with the kit as indicated. The inset shows milk from mouse #wt1-26 in dilutions as indicated. The dilution of .1 refers to a final concentration of 10% milk. In each dilution of transgenic milk, samples were supplemented with negative mouse milk in order to keep the final concentration at 10%. All points of the control curve and the experimental (inset) curve have the background value (the value determined for negative mouse milk) subtracted.

could be due to many factors. Preliminary data indicate that variation in t-PA expression levels among transgenic mice containing WAP-tPA may be considerable, suggesting that the chromosomal integration site may play a key role in establishing levels of expression from this construction. In fact, one transgenic mouse (not shown) appeared to express virtually no t-PA in the milk. Thus, analysis of additional animals may identify those which produce more t-PA. In addition, intragenic and/or noncoding 5' and 3' sequences from the WAP gene, missing from the construction introduced into mice in these experiments, may play important roles in RNA stability. Considerable work remains to be done to configure the t-PA expression vector for maximal expression.

We demonstrate here that a foreign protein, human tissue plasminogen activator, can be secreted into the milk of transgenic mice under the control of a mammary-specific promoter. Thus, concerns that foreign proteins produced in the mammary gland might not be secreted, accurately processed, or be sufficiently stable in milk appear to be reduced by these results. The advantages of producing foreign proteins in this manner include the fact that milk is well characterized biochemically and that many of the genes encoding key milk proteins have been cloned. In addition, many milk-specific genes are expressed in the lactating mammary gland at high levels under hormonal control and in a tissue-specific manner. Thus, with expression cassettes similar to the one described here, it should be possible to target precisely foreign gene expression to the lactating mammary epithelium. Factor IX and t-PA have been produced in the blood of transgenic mice^{14,15}; the ability to produce these proteins in milk would facilitate their collection. The ultimate goal of our experiments is to express foreign proteins in the milk of farm animals. Since production of transgenic farm animals has been achieved¹⁶, this presents a reasonable possibility. Although many technical hurdles remain, the data presented here demonstrate that transgenic animals may become an attractive alternative for future production of genetically engineered biologically active proteins.

EXPERIMENTAL PROTOCOL

Construction of expression vector. A Hind-III site was added at the 5' end of the 2.6 kb WAP promoter sequence (Fig. 1, Ref. 5) by digestion at the single EcoRI site in the WAP promoter, filling in with Klenow and dNTPs, and ligation of Hind-3 linkers, all by standard protocols. The t-PA cDNA (Fig. 1, SV40 termination/polyadenylation (■) cassette (see Ref. 11) was inserted into the polylinker region of the WAP promoter vector as a KpnI-Bam HI fragment.

Generation and analysis of transgenic animals. To purify the eukaryotic sequences for microinjection, WAP-tPA was digested with Hind-3 and BamHI, the fragments separated by gel electrophoresis, and the 4.9 kb fragment purified by binding to glass filter fiber papers¹⁷. After elution and concentration by ethanol precipitation, the DNA was suspended for microinjection in 10mM Tris, .05 mM EDTA, pH 7.5 at a concentration of .5 ng/microliter. The regulatory/coding restriction fragment from pWAP-tPA was microinjected into one cell fertilized embryos as described previously¹⁸. At four weeks of age, tail sections were taken from mice born from these injections, digested with proteinase K, phenol-chloroform extracted, then digested with various restriction enzymes. DNA was electrophoresed on a Tris-borate gel, blotted to nitrocellulose, and hybridized with a probe consisting of the entire coding region of t-PA cDNA (see bold line under the restriction map of Fig. 1). Labeling was done by extension of random hexamers to a specific activity of 1×10^9 cpm/ μ g.

Calcium phosphate transformation. MCF7 cells were plated in 100 mm dishes at densities of 5×10^6 or more per dish at least one day prior to transfection. Transfections were performed as described previously¹⁹ and transient supernatants collected 48 hours after transfection were assayed for t-PA.

Fibrin clot lysis assay. The fibrin clot assay measures the ability of t-PA to digest fibrinogen matrices which are laid down

in a background of agarose, thrombin and plasminogen within the wells of a plate²⁰. A small hole is bored through the agarose mixture upon hardening and 25 microliters of the samples are loaded into each of the holes. As t-PA diffuses into the agarose, clearing of the fibrinogen is evident visually and the amount of clearing is directly proportional to the amount of active t-PA. These assays are extremely sensitive and reproducible.

ELISA assay: Assays were performed with the IMUBIND ELISA kit produced by American Diagnostica Inc. The assay is a double antibody sandwich in which the primary antibody is a goat antiserum raised against t-PA from human uterus and the second antibody is a peroxidase conjugated anti-t-PA IgG. The standard curves were performed in negative mouse milk diluted to a final concentration of 10% with PBS, to which was added t-PA supplied with the kit.

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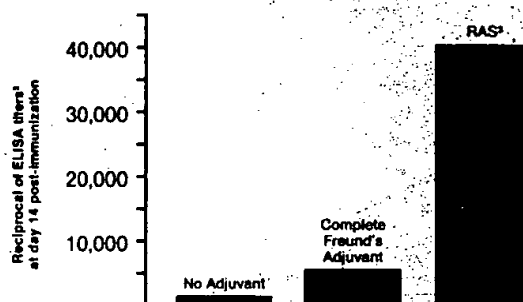
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ELISA Activity of Serum from BALB/c Mice Immunized with 25ug Bovine Galactosyltransferase* Alone or with Adjuvants



* Mice were injected with 25 ug of bovine galactosyltransferase.

† ELISA titers were determined with a solid-phase plate assay according to standard methods.

‡ Monophosphoryl Lipid A (MPL) + Trehalose Dimycolate (TDM)

J.T. Ulrich, unpublished data



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